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ning of each regular issue of the PCT Gazette.*(54) Title: **NUCLEOTIDE ANALOGUES**

(57) Abstract: The invention relates to nucleosides comprising a reporter moiety which also functions to limit polymerase activity, characterised in that the reporter moiety is attached to the nucleoside through a linkage group cleavable by a hydrolase enzyme wherein the hydrolase enzyme is selected from the group consisting of esterases, phosphatases, peptidases, penicillin amidases, glycosidases and phosphorylases.

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NUCLEOTIDE ANALOGUES

Field of Invention

- 5 The present invention relates to nucleoside and nucleotide analogues. In particular, the invention relates to nucleotide analogues comprising enzyme hydrolysable linkage groups attaching reporter moieties to the nucleotide.

Background of the Invention

- 10 Recent improvements in DNA sequencing techniques have sought to meet the increasing demands of large scale sequencing. Increasingly, methods in which the template nucleic acid molecules are attached to a solid surface are being developed (see, for example, US 5,302,509 and US 5,547,839). Such methods dispense with the
15 need for an electrophoretic separation step and, with the use of optical detection technologies (see, for example, Nie *et al.* Annu. Rev. Biophys. Biomol. Struct. 1997, 26: 567-96), aim to allow sequencing information at the level of a single molecule to be obtained. This has the further potential for multiple samples to be analysed simultaneously.

- 20 One example of such methods is Base Addition Sequencing Scheme (BASS) (see, for example, Metzker *et al.*, Nucleic Acids Res 1994, Vol.22, No.20; p. 4259-4267). BASS is a method involving the incorporation of nucleotide analogues which have been modified so as to comprise a blocking group which terminates DNA synthesis. A
25 primer is annealed to a template bound to a solid support and sequence data obtained by repetitive cycles of incorporation of modified nucleotides. At each cycle, the incorporated base is identified *in situ* before being deprotected to remove the blocking group and allow the next cycle of DNA synthesis.

- 30 Methods such as BASS rely on the use of nucleotide analogues that possess polymerase enzyme blocking (or terminator) groups at the 3' hydroxyl position of the sugar on the nucleotide. Typically, the blocking group is a combined terminator and label/reporter moiety such that the incorporated nucleotide can be detected while the bulky label or reporter moiety itself fulfils the role of blocking a polymerase from any

further DNA synthesis. Conveniently, as the terminator group is also the reporter moiety, a single reaction allows simultaneous removal of both functions thus allowing subsequent DNA synthesis and for incorporation of the next base to be read.

5 In order to allow subsequent rounds of DNA synthesis, these polymerase enzyme blocking groups are, typically, attached to the nucleotide via a linking group in such a way that they can be removed. However, conventional sequencing strategies require high temperatures of cycling (typically approximately 95°C or above) which are associated with pH changes in the reaction mixture. Such conditions can cause
10 reactivity of certain chemical bonds. Accordingly, the coupling methods for attaching blocking and labelling groups to nucleotides which have been used to date have focused on using those linking groups which can withstand changes in chemical conditions (such as temperature and pH). For example, the blocking and label groups can be attached via photosensitive linkage groups and thus cleavable by light
15 irradiation (i.e. photochemical means, see, for example, WO 93/05183) or via chemical means.

WO 01/92284 describes nucleotides which comprise both a reporter moiety and a polymerase blocking group in which the reporter moiety does not also act as a
20 polymerase enzyme blocking group. The polymerase enzyme blocking group is attached to the sugar by means of an enzyme cleavable linker.

Labeled reagents for nucleic acid sequencing are disclosed in EP 0252683. Igloi (BioTechniques, 1996, 21, 1084-1092) reports nucleotide-dye derivatives with utility
25 in sequencing. In both cases, stable propynyl amide linking groups are used to attach the fluorescent dye to the base of these reagents.

Similarly, stable propynyl linking groups are used in dye-labelled nucleotides in WO 97/ 00967 and WO/ 88 10264.

30

The use of known nucleotide analogues suffers from a number of disadvantages.

Known methods of removing the reporter/terminator groups require repeated insult by reactive chemicals or irradiation which can result in damage to the template DNA strand through reactions such as base transformation, crosslinking, or depurination.

- 5 Furthermore, by attaching the bulky reporter moiety in the 3' position of the nucleotide, the ability of the DNA polymerase to recognise or tolerate the nucleotide may be reduced. In addition to being poorly incorporated, modified nucleotides may be inactive (i.e. not incorporated), inhibitory (i.e. inhibit DNA synthesis) or may result in an alteration of the polymerase enzyme fidelity.

10

Any one of, or a combination of, these effects will result in a reduced accuracy in the sequence data obtained and, in particular, a decreased signal-to-noise ratio will be found on detection. Moreover, this means that the amount of sequence data that can be obtained from successive rounds of enzyme incorporation and cleavage is limited.

- 15 For example, if a combined error of approximately 3% in incorporation and cleavage were to accumulate, the result would be that sequence could only be obtained from 5 bases or fewer of the template DNA before the decreased signal to noise ratio made further sequencing impractical.

- 20 Accordingly, there is a need for improved nucleotide analogues. Such analogues may have one or more of the following attributes: tolerated by polymerases; stable during the polymerization phase; and the reporter groups can be removed efficiently under conditions which minimise damage to the template strand or template-primer complex. Preferably, the improved analogues display more than one of these features
- 25 and most preferably they display all of these features.

- It is thus an object of the invention to provide nucleoside and nucleotide analogues to which reporter moieties, which also limit polymerase-mediated incorporation, are attached via enzyme hydrolysable linkage groups. It is also an object of the invention
- 30 to provide nucleoside and nucleotide analogues comprising reporter groups which are removable on enzyme hydrolysis of a labile moiety attached to the linkage groups of the analogues. Such nucleotide analogues are most suitable for using in sequencing reactions which involve an isothermic reaction and therefore do not involve exposure of the nucleotide analogues to high temperatures and to undesirable variations in

chemical conditions. Under the conditions of suitable sequencing reactions, including array-based sequencing technologies (such as BASS), enzyme-cleavable groups will be essentially stable. The use of enzyme hydrolysable linking groups removes the need for harsh, template-damaging treatments to remove the reporter moieties.

Description of the Invention

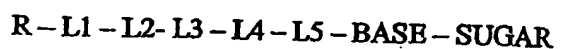
The present invention describes the use of linkage groups, cleavable by enzyme hydrolysis, to attach reporter moieties to nucleosides and nucleotides.

Accordingly, in a first aspect, the invention provides a nucleoside comprising a reporter moiety which also functions to limit polymerase activity, characterised in that the reporter moiety is attached to the nucleoside through a linkage group cleavable by a hydrolase enzyme, wherein the hydrolase enzyme is selected from the group consisting of esterases, phosphatases, peptidases, penicillin amidases, glycosidases and phosphorylases.

In contrast to enzyme cleavable nucleosides of the prior art, the reporter moiety serves the dual function of providing a detectable label and of limiting polymerase enzyme activity. Thus nucleosides of the present invention do not comprise a separate reporter moiety and a separate polymerase enzyme blocking group.

A hydrolase is defined as any member of the class of enzymes that catalyse the cleavage of a chemical bond with the addition of water.

In a second aspect, the invention provides a compound of Formula I:



(I)

wherein R is a reporter moiety

L1 and L5 are optional linkage groups each containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S.

5 L2 and L4 are optional linkage groups comprising 1 or more amino acid residues.

L3 is a linkage group that is susceptible to enzymic hydrolysis by a hydrolase enzyme, wherein hydrolytic cleavage may be within the group or adjacent to the group and
10 characterised in that the hydrolase enzyme is selected from the group consisting of esterases, phosphatases, peptidases, penicillin amidases, glycosidases and phosphorylases.

Suitably, enzymic hydrolysis of the linkage group L3 produces an unstable moiety
15 which undergoes chemical hydrolysis.

Suitable bases comprise purines or pyrimidines and, in particular, any of the bases A, C, G, U and T or analogues thereof.

20 Suitably, the sugars comprise ribose or deoxyribose or analogues thereof. Thus ribonucleotides and deoxyribonucleotides are envisaged together with other nucleoside analogues.

Suitably, a mono-, di- or tri- phosphate group is attached to the sugar. In a
25 particularly preferred embodiment, a triphosphate group is attached to the sugar.

In a preferred embodiment, the composite linker group L1 to L5 may be a chain of 10 to 200 bond lengths and may include atoms selected from carbon, nitrogen, oxygen and sulphur atoms, the linker group may be rigid or flexible, unsaturated or saturated
30 as is well known in the field. The composite linker group may further incorporate one or more amino acids joined by peptide bonds. The incorporation of amino acids can be through the incorporation of amino acid monomers or oligomers using standard amino acid chemistry (see, for example, "Synthetic Peptides - A Users Guide" Ed. G. A. Grant; 1992).

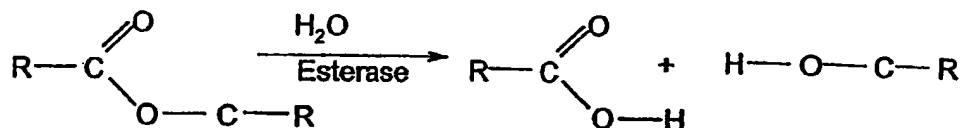
Hydrolysis of L3 by a hydrolytic enzyme selected from the group consisting of esterases, phosphatases, peptidases, glycosidases, penicillin amidases and phosphorylases results in the polymerase enzyme reporter moiety (R) becoming detached from the compound.

In a preferred embodiment, the linkage group L3 is cleavable by hydrolase enzymes selected from the group consisting of esterases, phosphatases, peptidases, glycosidases and phosphorylases. Preferred peptidases include subtilisin, proteinase K, elastase, neprilysin, thermolysin, papain, plasmin, trypsin, enterokinase and urokinase. Suitable enzymes are those that are reactive under mild conditions (see Handbook of Proteolytic Enzymes, Barrett *et al.*, ISBN 0-12-079370-9). In a particularly preferred embodiment, the enzyme-hydrolysable group is cleavable by penicillin amidase.

Preferably, L3 is a peptide selected from the group consisting of alanine-alanine-alanine, alanine-alanine-leucine, glycine-leucine-serine, glycine-serine-alanine-alanine-leucine and glycine-alanine-glycine-leucine.

Esterases catalyse the general reaction set out below in Reaction Scheme 1:

Reaction Scheme 1



Thus, in a further preferred embodiment of the second aspect, L3 comprises an ester group.

Non-specific esterase activity is associated with a number of enzyme systems. This activity has been associated with both physiological function and drug metabolism. Such a non-specific carboxylesterase activity can be used to modify molecules *in*

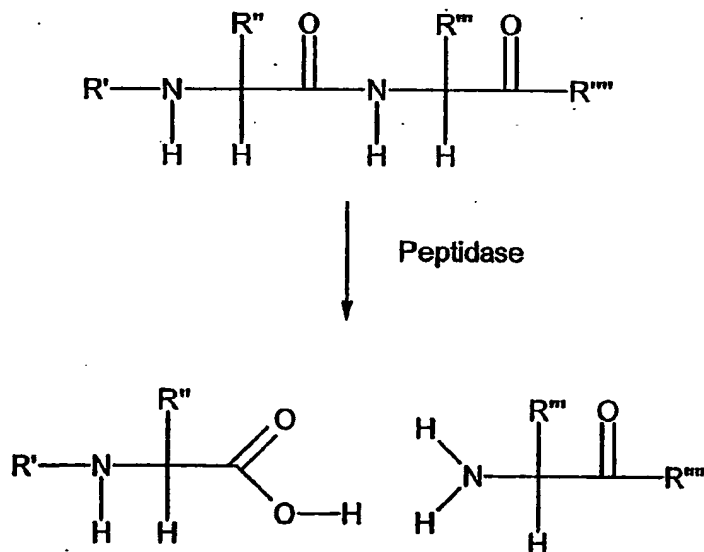
vitro. However, the lack of stability of carboxyesters at moderately high pH and elevated temperatures can make them unsuitable for generating stable reagents for nucleic acid applications.

- 5 In a preferred embodiment highly stable peptide bonds are utilised as specifically cleavable groups. The linkage groups may be digested with a suitable peptidase as shown in Reaction Scheme 2 to remove the reporter moiety without damaging the template strand or the template/primer complex. Following deprotection, further DNA synthesis can take place leading to the next cycle of labelled analogue addition.

10

Where R' and R'' both represent one or more amino acid residues, then peptidases catalyse the following general reaction set out in Reaction Scheme 2:

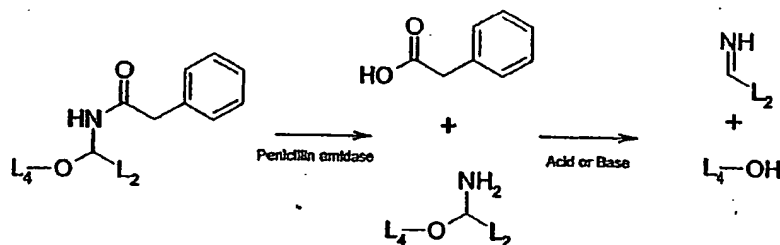
15 Reaction Scheme 2



- 20 Suitably, enzymic hydrolysis of the linkage group L3 produces an unstable moiety which undergoes chemical hydrolysis.

A linker may be assembled such that L3 comprises a moiety that is not part of the linker backbone and is not directly bonded to the base or the reporter groups (through, for example, linkage groups L2 or L4), which may be enzymatically hydrolysed to a chemically unstable form. Thus, on removal of such a moiety by a hydrolytic enzyme, the unstable linkage group formed rapidly undergoes chemical hydrolysis to facilitate cleavage of the linker. This is distinct from direct enzymatic cleavage of the linker by the enzyme in that the covalent bonds broken by the enzyme do not form part of the contiguous chain of covalent bonds comprising the linker between the nucleoside and the label. An example of this type of "remote cleavage" would be when L3 consists of an N-phenylacetyl amino acetal. The enzyme penicillin amidase specifically recognises the phenylacetyl portion of the molecule and hydrolyses the amide bond, giving phenyl acetic acid and a hemiaminal (see, for example, WO 97/20855). The hemiaminal is highly susceptible to acid or base catalysed hydrolysis in the absence of the enzyme. Thus if L3 comprises such a unit, treatment with penicillin amidase would render the linker chemically unstable, resulting in cleavage on base or acid catalysed hydrolysis. This is depicted in Reaction Scheme 3:

Reaction Scheme 3



20

Other systems that work on this principle could be devised and incorporated into the design of any linker. The particular advantage of this type of linker cleavage is that the enzyme recognition and cleavage site is remote from the linker cleavage site and may be less affected by the proximity of the other components of the linker, the label or the nucleoside.

25

Accordingly, in a particularly preferred embodiment, L3 comprises a penicillin amidase cleavage site. Penicillin amidase is also known as penicillin aminohydrolase (EC 3.5.1.11).

- 5 Suitable methods for attaching a linker comprising an enzyme cleavable group to a base moiety are described, for example, in Cavallaro *et al.* Bioconjugate Chem. 2001, 12, 143-151. Further methods are described in Langer *et al.*, Proc Natl Acad Sci USA, 1981, 78, 6633-6637; Livak *et al.*, Nucleic Acids Res, 1992, 20, 4831-4837 and Gebeyehu *et al.*, Nucleic Acids Res, 1987, 15, 4513-4534.

10

- A suitable reporter moiety, R, may be any one of various known reporting systems. It may be a radioisotope by means of which the nucleoside analogue is rendered easily detectable, for example ^{32}P , ^{33}P , ^{35}S incorporated in a phosphate or thiophosphate or H phosphonate group or alternatively ^3H or ^{14}C or an iodine isotope. It may be an isotope detectable by mass spectrometry or NMR. It may be a signal moiety e.g. an enzyme, hapten, fluorophore, chromophore, chemiluminescent group, Raman label, leucodye, electrochemical label, or signal compound adapted for detection by mass spectrometry.

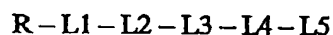
- 20 In a preferred embodiment, the reporter moiety has fluorescent properties and can be detected using a sensitive fluorescence detector. It may be a fluorophore, for example, selected from fluoresceins, rhodamines, coumarins, BODIPY® dyes, phenoxazine dyes, cyanine dyes and squarate dyes (described, for example, in WO 97/40104). Preferably, the dyes are acridone derivatives, as described in patent applications GB 0113435.2 and GB0113434.5. Most preferably, the reporter moiety is a cyanine dye. The Cyanine dyes (sometimes referred to as "Cy dyesTM"), described, for example, in US Patent 5,268,486, are a series of biologically compatible fluorophores which are characterised by high fluorescence emission, environmental stability and a range of emission wavelengths extending into the near infra-red which can be selected by
30 varying the internal molecular skeleton of the fluorophore.

In a preferred embodiment, the modified nucleotide remains compatible with elongation enzymology, i.e. it can still be incorporated by a polymerase. Procedures for selecting suitable nucleotide and polymerase combinations will be readily adapted

from Metzker *et al.*, Nucleic Acids Res 1994, Vol. 22, No. 20, 4259-4267. In particular, it is desired that a selected polymerase be capable of selectively incorporating a nucleotide.

- 5 In another preferred embodiment, the reporter group, R, restricts further elongation of the polymer to a limited number of additions by a polymerase once the nucleotide of the present invention has been incorporated by a selected polymerase in specified polymerase enzyme conditions.
- 10 The invention further provides a chemical intermediate selected from the group consisting of: 5-N-(N-Trifluoroacetyl- β -alanyl)propargylamino-2'-deoxyuridine; 5-N-(β -alanyl) propargylamino-2'-deoxyuridine; 5-N-(N-Fluorenylmethyloxycarbonyl-Gly-Gly-Leu- β -alanyl)propargylamino-2'-deoxyuridine; 5-N-(Gly-Gly-Leu- β -alanyl)propargylamino-2'-deoxyuridine and 5-N-[N-(6-Fluorescein-5(and-
15 6)carboxamidohexanoyl)-Gly-Gly-Leu- β -alanyl]-propargylamino-2'-deoxyuridine.

In a third aspect, the invention provides a chemical intermediate of Formula II



20

(II)

wherein R is a reporter moiety

25

L1 and L5 are optional linkage groups each containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S,

30

L2 and L4 are optional linkage groups comprising 1 or more amino acid residues, and

L3 is a linkage group that is susceptible to enzymic hydrolysis by a peptidase enzyme. Chemical intermediates of Formula II are of use as dye-linker groups in the synthesis of compounds of Formula I.

- 5 In a preferred embodiment, L3 is selected from the group consisting of alanine-alanine-alanine, alanine-alanine-leucine, glycine-leucine-serine, glycine-serine-alanine-alanine-leucine and glycine-alanine-glycine-leucine.

- Preferably, the reporter R (or dye) is selected from the group consisting of of
10 fluoresceins, rhodamines, coumarins, BODIPY® dyes, phenoxazine dyes, cyanine dyes, acridone dyes and squarate dyes.

- In a fourth aspect, the invention provides a compound comprising 5-N-[N-(6-Fluorescein-5(and-6) carboxamidohexanoyl)-Gly-Gly-Leu- β -alanyl]-propargylamino-
15 2'-deoxyuridine triphosphate.

- In a fifth aspect, the invention provides a set of nucleotides characterised in that the set contains at least one compound of Formula 1 having a mono-, di- or triphosphate group attached to the sugar. Preferably, the set comprises each of the four natural
20 bases A, G, C and T and analogues thereof.

- In a preferred embodiment of the fifth aspect the set of nucleotides further comprises at least two compounds as described above having different bases, characterised in that each compound has a different reporter moiety, R. Thus, for example, the set of
25 nucleotides may comprise compounds with bases A and G, wherein the compound with base, A, has a first reporter moiety (R^1) and the compound with base, G, has a second reporter moiety (R^2), and the first and second reporter molecules are distinguishable from each other.

- 30 In another preferred embodiment of the fifth aspect, the set of nucleotides comprises four compounds as described above characterised in that each compound has a different base such that each of the bases A, G, C and T, or analogues thereof, are

present and each of the four compounds has a reporter moiety which is distinguishable from the reporter moiety of each of the compounds having the other three bases.

In a sixth aspect, the invention provides a method for nucleic acid molecule

5 sequencing comprising the steps of:

- a) immobilising a complex of a primer and a template to a solid phase
- b) incubating with a polymerase in the presence of a compound of Formula I having a mono-, di- or triphosphate group attached to the sugar.

10 In one embodiment of the sixth aspect, the complex of primer and template can be preformed by incubation under appropriate hybridisation conditions before immobilising the complex onto a solid phase. In another embodiment, the primer or the template can be immobilised onto a solid phase prior to formation of the complex by introduction of the appropriate hybridisation partner (i.e. template or primer,
15 respectively). In yet another embodiment, the complex immobilised onto the solid phase can be a single nucleic acid molecule comprising both "primer" and "template"; for example, the immobilised poly- or oligo- nucleotide can be a hairpin structure.

Suitable polymerases are enzymes that perform template-dependent base addition
20 including DNA polymerases, reverse transcriptases and RNA polymerases. Suitable native or engineered polymerases include but are not limited to T7 polymerase, the Klenow fragment of *E. coli* DNA polymerase I which lacks 3'-5' exonuclease activity, *E. coli* DNA polymerase III, SequenaseTM, ϕ 29 DNA polymerase, exonuclease-free Pfu, exonuclease-free VentTM polymerase, Thermosequenase, Thermosequenase II,
25 Tth DNA polymerase, Tts DNA polymerase, MuLv Reverse transcriptase or HIV reverse transcriptase. The selection of an appropriate polymerase depends on the interaction between a polymerase and the specific modified nucleotide (as described by Metzker *et al.*, Nucleic Acids Res 1994, Vol.22, No.20; p. 4259-4267).

30 Nucleotides comprising hydrolase-cleavable linkage groups such as carboxyl ester attachment groups are suitable for use in sequencing reactions used in array based sequencing, such as BASS. Such reactions are isothermal, unlike cycle sequencing, so allowing much better control of reaction conditions. In particular, the sequencing

reaction takes place at relatively low temperatures (typically less than 70°C) thus enabling enzyme-cleavable linkage groups, such as the carboxyl ester attachment, to remain stable under these sequencing reaction conditions. Accordingly, polymerases which may be useful in the sixth aspect of the invention include thermostable
5 polymerases and non-thermostable polymerases.

In a preferred embodiment of the sixth aspect, the method further comprises the steps of :

- c) detecting the incorporation of a compound of Formula I having a mono-, di- or
10 triphosphate group attached to the sugar
- d) incubating in the presence of enzyme under suitable conditions for enzymatic cleavage of the enzyme-cleavable group L3

Preferably, the hydrolase enzyme is selected from the group consisting of esterases,
15 phosphatases, peptidases, penicillin amidases, glycosidases and phosphorylases.

Suitable conditions for enzyme hydrolysis of the enzyme-cleavable groups will depend on the nature of the enzymes involved. Enzymes such as carboxyesterases are active under a broad range of conditions and do not require co-factors. Commercially
20 available carboxyesterases will hydrolyse esters under mild pH conditions of between pH 7.0 and pH 8.0. e.g. 0.1M NaCl, 0.05M Tris.HCl, pH 7.5.

Suitable peptidases may be selected from the group consisting of subtilisin, proteinase K, elastase, neprilysin, thermolysin, papain, plasmin, trypsin, enterokinase and
25 urokinase. Suitable conditions for cleavage by peptidases are exemplified in Example 5 below.

In another embodiment of the sixth aspect, the method further comprises:

- e) repeating steps a)-d)

30

In a preferred embodiment of the sixth aspect, the enzyme in step d) is penicillin amidase.

In a preferred embodiment of the sixth aspect, the incorporation of the compound is determined by the detection of a single reporter group attached to the compound.

Briefly, sequencing reactions using modified nucleotides in accordance with the second aspect of the invention may be performed as follows. Primer template complexes are immobilised to a solid surface and contacted with modified nucleotides in the presence of a suitable buffer also containing a polymerase, such as Klenow fragment of *E. coli* DNA polymerase I which lacks 3'-5' exonuclease activity, and a commercially available pyrophosphatase. The reaction is incubated under suitable conditions for a polymerase-mediated base addition reaction followed by the removal of non-incorporated nucleotides and enzymes by washing with a wash buffer. Suitably, the wash buffer contains a buffering agent, such as an organic salt, to maintain a stable pH of approximately pH 6 to pH 9 and possibly also comprises monovalent or divalent cations and a detergent so as to eliminate non-covalently bound molecules from the solid surface. Where the modified nucleotides comprise a fluorescent reporter molecule, incorporated nucleotides are detected by measuring fluorescence. Following identification, the templates are contacted with a buffered solution containing an excess of a protein displaying the appropriate enzyme activity and incubated under conditions for enzyme cleavage activity. For example, where the enzyme-cleavable group linking the reporter moiety to the nucleotides is a peptide group, the solution contains an excess of a protein displaying peptidase activity. Following enzyme activity, the soluble products of enzymatic cleavage are eliminated by washing as above. Following the washing step, the immobilised template is washed with an excess of buffer used for the polymerase reaction and the steps of polymerase-mediated base addition, detection of incorporated nucleotide and enzyme-cleavage activity are repeated to obtain further sequence data.

Specific Description

For the purposes of clarity, certain embodiments of the present invention will now be described with reference to the following figures and examples:

Figure 1 (Example 1) shows a reaction scheme for synthesising a compound of Formula I.

Figure 2 (Example 2) depicts enzyme cleavage of a compound of formula I.

Figure 3 (Example 3) shows a reaction scheme for the synthesis of a nucleotide with a penicillin amidase cleavable linker.

Example 4 describes the incorporation of a compound of Formula I by a DNA
5 polymerase.

Example 5 describes protease mediated cleavage of FamHex-GGL- β -A-2'dU

Example 6 describes the preparation of dye-labelled nucleosides with protease
cleavable linkers

10 Example 1

A reaction scheme for the synthesis of an example of a compound of Formula I
containing a peptide-based linker is set out in Figure 1.

15 i) 5-N-(N-Trifluoroacetyl- β -alanyl)propargylamino-2'-deoxyuridine (2)

5-Propargylamino-2'-deoxyuridine (1) (1.3g, 4.6mmol) and N-trifluoroacetyl- β -alanine succinimidyl ester (1.29g, 4.6mmol) were dissolved in DMF (10mL) at ambient temperature. Triethylamine (0.46g, 0.6mL, 4.6mmol) was added and the
20 solution stirred overnight at ambient temperature. The solvent was then removed under vacuum. The residue was then re-dissolved in dichloromethane : methanol (1:1) and eluted through a flash silica gel column (dichloromethane : methanol, 9:1). Removal of solvent from the appropriate fractions (R_f 0.1, dichloromethane : methanol 9 : 1) afforded the title compound as a white foam (0.7g, 34%). ^1H NMR (300MHz, d_6 -DMSO) δ 11.62 (1H, s, $\text{N}^3\text{-H}$), 9.48 (1H, t, br, CF_3CONH), 8.48 (1H, t, J 5.4Hz, propargyl NH), 8.15 (1H, s, H-6), 6.09 (1H, app t, J 6.6Hz, H-1'), 5.25 (1H, d, J 4.5, 3'-OH), 5.10 (1H, t, J 4.5Hz, 5'-OH), 4.22 (1H, m, H-4'), 4.07 (2H, d, J 5.1Hz, propargyl CH₂), 3.77 (1H, m, H-3'), 3.61 – 3.51 (2H, m, H-5'), 3.40 – 3.33 (2H, β -ala CH₂CONH part obs. by H₂O), 2.37 (2H, t, J 6.9Hz, β -ala CH₂NHTFA), 2.10 (2H, dd, J 4.8Hz, H-2'); ^{13}C NMR (75.45MHz, d_6 -DMSO) δ 169.37, 161.61, 156.19 (q, J^2_{CF} 35.9Hz), 115.85 (q, J^1_{CF} 286.5Hz), 98.06, 89.45, 87.62, 84.70, 74.44, 70.23, 70.13, 61.02, 35.78, 33.80, 28.58, 25.20, 8.75; MS (ES+) m/z 449 (M+H)⁺, 466 (M+H₂O)⁺.
30

ii) 5-N-(β -alanyl)propargylamino-2'-deoxyuridine (3)

5-N-(*N*-Trifluoroacetyl- β -alanyl)propargylamino-2'-deoxyuridine (2) was dissolved in concentrated aqueous ammonia at ambient temperature. The solution was allowed to stir overnight at ambient temperature. The solvent was then removed under vacuum to give the title compound as a pale yellow foam (0.62g, 100%). ¹H NMR (300MHz, d₆-DMSO) δ 8.61 (1H, t, *J*5.1Hz, propargyl NH), 8.16 (1H, s, H-6), 6.09 (1H, app t, *J*6.6Hz, H-1'), 4.21 (1H, m, H-4'), 4.10 (2H, d, *J*5.1Hz, propargyl CH₂), 3.79 (1H, m, H-3'), 3.59 (2H, m, H-5'), 2.96 (2H, t, *J*6.9Hz, β -ala CH₂), 2.44 (2H, t partly obs., β -ala CH₂), 2.10 (2H, m, H-2'); ¹³C NMR (75.45MHz, d₆-DMSO) δ 169.23, 161.61, 149.41, 143.76, 97.98, 89.25, 87.64, 84.74, 74.60, 55.46, 35.31, 32.36, 28.62, 25.20; MS (ES+) *m/z* 353(M+H)⁺.

iii) 5-N-(*N*-Fluorenylmethyloxycarbonyl-Gly-Gly-Leu- β -alanyl)propargylamino-2'-deoxyuridine (4)

5-N-(β -alanyl)propargylamino-2'-deoxyuridine (3) 0.1g (0.28mmol) and *N*-fluorenylmethyloxycarbonyl-Gly-Gly-Leu succinimidyl ester (0.17g, 0.3mmol) were weighed into a round bottom flask and then dissolved in anhydrous DMF (1mL). Triethylamine (0.061g, 0.08mL, 0.6mmol) was then added and the reaction mixture stirred at ambient temperature. After 2.5 hours the solvents were removed under vacuum and the residue redissolved in dichloromethane – methanol (9:1) and eluted with dichloromethane – methanol (9:1 then 8:2). Collected and removed solvent from fractions containing R_f 0.5 material (8:2 dichloromethane : methanol). Obtained the title compound as a white solid 0.04g (18%). ¹H NMR (300MHz, d₆-DMSO) δ 11.60 (1H, s, br, N³-H), 8.38 (1H, t, amide NH), 8.15 (1H, s, H-6), 7.95 (1H, t, amide NH), 7.90 (1H, d, amide NH), 7.88 (2H, d, *J*7.5Hz, Fmoc), 7.70 (2H, d, *J*7.5Hz, Fmoc), 7.62 (1H, t, amide NH), 7.41 (2H, t, *J*7.5Hz, Fmoc), 7.30 (2H, t, *J*7.5Hz, Fmoc), 6.09 (1H, app t, *J*6.6Hz, H-1'), 5.24 (1H, d, br, 3'-OH), 5.09 (1H, t, 5'-OH), 4.29 – 4.18 (4H, m), 4.05 (2H, m), 3.79 – 3.54 (5H, m), 2.25 (2H, m, β -ala CH₂), 2.10 (2H, m, H-2'), 1.51 (1H, sept., leucyl CH₂CH(CH₃)₂), 1.42 (2H, m, leucyl CH₂CH(CH₃)₂), 0.84 – 0.78 (6H, 2d, leucyl CH₂CH(CH₃)₂); MS (ES+) *m/z* 802(M+H)⁺.

iv) 5-N-(-Gly-Gly-Leu-β-alanyl)propargylamino-2'-deoxyuridine (5)

5-N-(N-Fluorenylmethyloxycarbonyl-Gly-Gly-Leu-β-alanyl)propargylamino-2'-deoxyuridine (4) was dissolved in anhydrous DMF at ambient temperature.

- 5 Piperidine was then added and the solution stirred at ambient temperature overnight. Solvents and volatile reagents were removed under vacuum. The product was used without further purification. MS (ES+) m/z 580(M+H)⁺.

10 v) 5-N-[N-(6-Fluorescein-5(and-6)carboxamidohexanoyl)-Gly-Gly-Leu-β-alanyl]-propargylamino-2'-deoxyuridine (6)

- 5-N-(-Gly-Gly-Leu-β-alanyl)propargylamino-2'-deoxyuridine (5) (11.2 μmol) and 6-(fluorescein-5(and 6)carboxamidohexanoic acid succinimidyl ester (0.098g, 16.8 μmol) were dissolved in DMF (0.2mL) at ambient temperature. Triethylamine
15 (3μL, 22.4 μmol) was added and the solution allowed to stand at ambient temperature overnight. Solvents and volatile reagents were removed under vacuum. The residue was washed with dichloromethane – methanol – acetic acid 90:9:1 to remove unreacted dye and residual triethylamine. The solid residue was then dissolved in methanol – water and purified by reverse phase HPLC (isocratic water – methanol 1:1
20 / C18 stationary phase). Obtained the title compound (5.3 μmol) as an orange powder after lyophilisation of the appropriate fractions. ¹H NMR (300MHz, d₄-MeOH) δ 8.47 (1H, s), 8.28 (1H, s), 8.20 – 8.00 (3H, m), 7.31 (1H, d), 7.10 – 7.00 (4H, m), 6.21 (1H, app t, H-1'), 4.6 (1H, m), 4.30 (2H, m), 4.21 (2H, d, propargyl CH₂), 3.9 – 3.6 (7H, m, glycyl α-H, H-5', leucyl α-H), 2.5 – 2.1 (6H, m, H-2', β-ala CH₂, caproamide
25 CH₂CONH), 2.75 – 1.4 (12H, caproamide CH₂, leucyl CH₂CH(CH₃)₂), 0.80 (6H, 2d, leucyl CH₂CH(CH₃)₂); MS (ES+) m/z 1051(M+H)⁺; UV λ_{max} 498nm (MeOH – NH₄OH, pH9).

30 vi) 5-N-[N-(6-Fluorescein-5(and-6)carboxamidohexanoyl)-Gly-Gly-Leu-β-alanyl]-propargylamino-2'-deoxyuridine triphosphate

5-N-(N-Fluorenylmethyloxycarbonyl-Gly-Gly-Leu-β-alanyl)propargylamino-2'-deoxyuridine (4) may be converted to a dye-labelled triphosphate by using established

triphosphate syntheses (for example, see K. Burgess & D. Cook., Chem. Rev. 2000, 100, 2047 – 2059 and references cited therein). The triphosphate of compound (4) could then be treated with piperidine under the same conditions as used to prepare compound (5) and then labelled as described above for the preparation of compound (6).

Example 2: Protease Mediated Cleavage of 5-N-[N-(6-Fluorescein-5(and-
6)carboxamidohexanoyl)-Gly-Gly-Leu- β -alanyl]-propargylamino-2'-deoxyuridine
(FamHex-GGL- β -A2'dU)

Figure 2 shows the hydrolytic cleavage of FamHex-GGL- β -A2'dU (6) by the proteolytic enzyme, subtilisin. Compound 6 is readily digested by subtilisin (Subtilopeptidase A, type VIII, Sigma Chemical Company, UK), which cleaves at the leucine residue, following 2 hours incubation at 37° C at pH 7.5 to yield the nucleoside and dye-labelled products shown.

Example 3: Synthesis of a Nucleotide with a Penicillin Amidase Cleavable linker

Figure 3 shows a reaction scheme for the preparation of a nucleotide with a penicillin amidase cleavable linker.

5-Hydroxymethyl-5',3'-di-*O-p*-toluyl-2'-deoxyuridine (7) (prepared using established procedures (T. Ueda, Chemistry of Nucleosides and Nucleotides, Vol. 1, Ed. L. B. Towensend) and *N*-[α -thioethyl-*N'*-trifluoroacetylaminopropyl benzamide] phenylacetamide (8) (Flitsch *et. al.*, Tetrahedron Letters 1998, 39, 3819 – 3822 and references cited therein; Flitsch *et. al.* WO 97/20855) may be combined in the presence of *N*-iodosuccinimide to give compound (9). Compound (9) may be converted to the intermediate (10) by treatment with sodium methoxide in methanol, followed by ethyl trifluoroacetate in methanol. Conversion of the nucleoside (10) to a triphosphate (11) may be achieved by using established triphosphate synthesis conditions (For examples see K. Burgess, D. Cook. Chem. Rev. 2000, 100, 2047 – 2059 and references cited therein). Labelling of the triphosphate with a reporter group may then be achieved by exposure of compound (11) to proprietary labelling reagents,

such as 6-[Fluorescein-5(and-6)-carboxamidohexanoic acid succinimidyl ester, in a suitably buffered aqueous solution to give (12).

Example 4: DNA polymerase assays

5

Incorporation of fluorescently labelled deoxynucleotide triphosphates by DNA polymerases

(i) Materials

10

The following oligonucleotides (Interactiva Biotechnologie, Germany) were used for assaying nucleotide incorporation:

Primer Sequence:-

15

SEQ. ID No 1:

5'- TAA CTC ATT AAC AGG ATC-3'

Template oligonucleotides:-

20

SEQ. ID No2:

ATTCGCGGTATTCTGGTATGAAGCTTTTAGATCCTGTTAATGAGTTAGTA

SEQ. ID No 3:

ATTCGCGGTATTCTGGTATGAAGCTTTAAGATCCTGTTAATGAGTTAGTA

25

SEQ. ID No 4:

ATTCGCGGTATTCTGGTATGAAGCTTAAAGATCCTGTTAATGAGTTAGTA

SEQ. ID No 5

30

ATTCGCGGTATTCTGGTATGAAAAAAAAAAGATCCTGTTAATGAGTTAGTA

Fluorescently labelled nucleotides were obtained from the following sources:-

Molecular Probes Inc:

Alexa Fluor 546-14-dUTP 1mM in TE

Alexa Fluor 568-5-dUTP 1mM in TE

Alexa Fluor 594-5-dUTP 1mM in TE

5 Nen, UK:

Fluorescein-12-dUTP

Coumarin-5-dUTP

Tetramethylrhodamine-6-dUTP

Texas Red-5-dUTP

10 Lissamine-5-dUTP

Naphthofluorescein-5-dUTP

Fluorescein Chlorotriazinyl-4-dUTP

Pyrene-8-dUTP

Diethylaminocoumarin-5-dUTP

15

Amersham Biosciences, UK:

Cy3 dUTP

Cy5 dUTP

20 (ii) Oligonucleotide labelling

Oligonucleotide 1 was labelled with [γ -33P]ATP (Amersham Biosciences) using T4 Polynucleotide Kinase (Amersham Biosciences) in accordance with the manufacturer's protocol.

25

(iii) Primer extension reactions

Each 20 μ l primer extension reaction, assembled in 0.5ml thermal cycling microfuge tube, contained at a final concentration 1x Thermosequenase™ buffer (Amersham Biosciences), 0.05mM 33P labelled oligonucleotide 1, 0.125mM template oligonucleotide, 0.125mM dye-labelled nucleotide and 0.165 units of enzyme. The reactions were denatured at 95°C for 1 minute before incubation at 48°C for 45 minutes.

30

The reactions were terminated by the addition of 5µl of stop buffer comprising 0.1%(w/v) Xylene Cyanol, 0.1%(w/v) Bromophenol blue and 80% Formamide. The reactions were heated to 90°C for 3 minutes then chilled on ice.

- 5 A 14% denaturing polyacrylamide gel in 1xTBE (Sequagel, Flowgen Ltd, UK and Sigma Chemical Co., UK, respectively) was pre-run at 40W constant power for 30min. in 1x TBE running buffer. An 8µl aliquot of denatured reaction was then applied to each lane and the samples electrophoresed for 1.5 hours at 40W constant power. The gel was exposed to a phosphor screen (Amersham Biosciences) for 1 hour
10 and the image detected on the Storm™ Imager (Amersham Biosciences) in accordance with the manufacturers guidelines.

(iv) Results

- 15 All the fluor-labelled nucleotides were incorporated by Thermosequenase. Templates 2, 3, 4 and 5 had 1, 2, 3, or 8 consecutive A residues respectively. The number of consecutive fluor-labelled nucleotides added was dependent upon the template and the fluor species and is summarised below:

20	Fluor	predominant product with templates 3,4 and 5
	Cy3	One addition, with minor two additions
	Cy5	One addition, with minor two additions
	Tetramethylrhodamine	One addition, with minor two additions
	Coumarin	One addition, with a comparable amount of template
25		with two additions
	Alexa 546-14-dUTP	One addition, with a comparable amount of template
		with two additions
	Fluorescein	One addition, with minor two additions
	Texas Red	One addition, with very slight two additions
30	Lissamine	One addition, with very slight two additions
	Alexa568-5-dUTP	One addition
	Alexa 594-5-dUTP	One addition

Even when the template contained three or more consecutive dA residues (oligonucleotide 4 and 5), the number of nucleotides added did not noticeably exceed two bases. The efficiency of the second base addition appeared to be dependent on the label attached. Some fluors such as Coumarin appeared to demonstrate greater efficiency in adding two consecutive bases.

Example 5: Protease mediated cleavage of 5-N-[N-(6-Fluorescein-5(and-6) carboxamido hexanoyl)-Gly-Gly-Leu- β -alanyl]-propargylamino-2'-deoxyuridine (6) (FamHex-GGL- β -A-2'dU)

A 10 μ g aliquot of the substrate FAMHEX-GGL- β -A 2'dU (compound 6 above) was dissolved in 0.1M sodium acetate buffer pH 7.5 containing 5mM calcium acetate. The substrate solution was digested at 37°C for 2 hours in 200 μ l in the presence of 0.5 units of subtilisin (Subtilopeptidase A, type VIII, Sigma Chemical Co. UK)

The resulting digest was ultrafiltered using a Microcon YM10 Concentrator (Milipore Ltd., UK) and the filtrate analysed by HPLC (Gilson 170, Gilson, UK) on a Sephasil™ peptide C-18 column (Amersham Biosciences, UK). Four major peaks of absorbance at 438 nm were observed in the substrate and product. These eluted at 29.8 (I), 32.6 (II), 26.7 (III) and 37.1 (IV) minutes on an acetonitrile gradient of 50% to 70%. The peaks were collected and analysed by MALDI-TOF mass spectrometry (Bruker Biflex II, Bruker, Germany). A matrix of 10mg/ml 3-hydroxycinnamic acid in acetonitrile and 1% TFA was used for the analysis. The mass spectrometer was calibrated with 100mM Bradykinin 1-7 (Sigma Chemical Co., UK).

The undigested substrate contained a predominant peak (I) that was shown by mass spectrometry to contain the intact substrate molecule. The remaining peaks corresponded to components of the intact molecule probably carried over from the synthesis. After substrate digestion with subtilisin, peak I was seen to diminish substantially and peak II demonstrated a corresponding increase in absorbance at 438nm. The latter peak was shown to contain a molecular ion of 717 that corresponded to a fluor-linker moiety resulting from the hydrolysis of the substrate at the peptide bond on the carboxyl side of the leucine residue. These observations were

consistent with the cleavage of the linker by the subtilisin enzyme. There was no observable change in peaks III and IV, suggesting that they do not act as substrates for the protease enzyme.

5 Example 6: Preparation of dye-labelled nucleosides with protease cleavable linkers

Linker motifs suitable for use in protease cleavable linkers were prepared and identified using the following procedures.

10 (i) General Library Amino Acid Coupling Procedure

A polystyrene resin loaded with 5-propargylamino-2'-deoxyuridine was prepared using standard solid phase chemical methods. The resin was distributed between 21 disposable filter vessels (10-20 mg per vessel). The filter tubes containing the resin
15 were placed on a vacuum manifold, DCM was added to swell the resin and the excess was drained off. An microtag was added to the vessel for identification purposes. Solutions of Fmoc-AA-OH (or Fmoc-Ahx-OH), DIC and HOBt in DCM/ DMF were prepared. The appropriate solution of activated amino acid was added, the reaction vessels, attached to the manifold, were placed horizontally on a flat bed shaker. The
20 vessels were agitated for 3-3.5 hours. The vessels were drained of the reaction mixture and the resin was washed with: DMF (5 x 1ml), DCM (5 x 1ml), MeOH (5 x 1ml), Et₂O (3 x 1ml), (volume of washings are shown per filter tube).

(ii) General Library Fmoc Deprotection Procedure

25 DMF was added to the vessels to allow the resin to swell. After draining off the DMF 20% piperidine in DMF was added and the vessels left to agitate for 1 hour. The deprotection mixture was drained off and the resin was washed with: DMF (5 x 1ml), DCM (5 x 1ml), MeOH (5 x 1ml), Et₂O (3 x 1ml).

30

Amino acid coupling and Fmoc deprotection procedures were repeated with different combinations of activated amino acids added to each tube to generate a library of compounds. Fmoc-Ahx-OH was the final amino acid to be added to all portions of

the resin. After final Fmoc deprotection the compounds were labelled with dye and cleaved from the solid support according to the procedures described below.

5 (iii) General Fluorophore Coupling Procedure

To the pre-swollen resin (DMF) was added a solution of fluorescein isothiocyanate isomer I and NEt₃ in DMF (1 ml). The resin was shaken overnight and was then washed with DMF (5 x 1 ml), DCM (5 x 1 ml), MeOH (5 x 1 ml) and Et₂O (5 x 1
10 ml).

(iv) General Cleavage Procedure

5% TFA in DCM (0.2 ml) was added to resin (8 to 14 mg) and left to stand for 2
15 minutes. DCM (0.3 ml) was added and the resin was left to stand for 3 minutes before the DCM/ TFA mixture was collected. 5% TFA in DCM (0.2 ml) was added and the resin left to stand for 2 minutes. A drop of MeOH and DCM (0.3 ml) was added and the resin was left to stand for 3 minutes. This cleavage procedure was repeated (x ~ 3) to ensure maximum release of compound. An average loading of 0.430 mmole/g
20 could be determined by HPLC.

(v) Protease Cleavage Assay

All compounds prepared were assayed with a selection of the preferred proteases to
25 determine the most suitable linker / protease combinations.

A solution of approximately 10µg of nucleoside in water (5µl) was mixed with 1 unit of protease (1 - 3µl depending on enzyme stock solution) in the appropriate buffer for the enzyme. Final volumes of solutions were made up to 200µl. Solutions were then held at enzyme optimum temperature for two hours and then centrifuged through
30 10,000D molecular weight size exclusion membranes [Amicon Microcon YM-10] to remove the enzyme. The appropriate protease substrates reactions and standard control solutions were also prepared and treated in the same manner. All solutions were analysed using C18 reverse phase h.p.l.c (0.1%TFA/water : 0.042%TFA/Acetonitrile, 95:5 – 0:100 linear gradient). Successful cleavage of the

linker was observed when several new products appeared on h.p.l.c., some of which possessed only the nucleoside chromophore.

5 (vi) Preparation of representative dye-labelled nucleotide triphosphate with a protease cleavable linker

Preparation of a dye labelled nucleotide triphosphate incorporating a protease cleavable linker was performed as described below after selection of a suitable linker
10 motif from the nucleoside library.

a) Preparation of Dye-Linker Group

The synthesis of an example of a dye-linker group, according to Formula II, is
15 described below.

Cy5 carboxylic acid (100mg, 0.17mmol) [Amersham Biosciences] and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (57mg, 0.19mmol) [Fluka] were weighed into an oven-dried round-bottom flask. Anhydrous dimethylsulfoxide
20 (250µl) [Aldrich] was then added to dissolve the solids. Neat diisopropylethylamine (32mg, 0.25mmol, 43µl) [Aldrich] was then added. The resulting solution was then stirred at ambient temperature for 4 hours. Thin layer chromatography (4:1 dichloromethane : methanol) showed the complete conversion of the starting material (Rf 0.2) to the N-hydroxysuccinimidyl ester (Rf 0.5). A solution of H2N-
25 GlyGlyLeu-OH (42mg, 0.17mmol) [Bachem] was prepared by heating the peptide in anhydrous dimethylsulfoxide at 60°C. The solution was then allowed to cool to ambient temperature and was then added immediately to the solution of the Cy5-carboxylic acid N-hydroxysuccinimidyl ester. The reaction mixture was stirred at ambient temperature overnight. Analysis by thin layer chromatography (4:1
30 dichloromethane : methanol) showed the complete conversion of the active ester (Rf 0.5) to a new product which was immobile on thin layer chromatography. The majority of the solvents were then removed under vacuum to give the crude product as a dark blue oil. The oil was redissolved in methanol (80µl). Half of the solution was then added to the top of a flash silica gel column and then eluted with 79:20:1

dichloromethane : methanol : acetic acid. Fractions were collected and those containing the predominant blue coloured component were pooled. The solvent was then removed under vacuum to give the pure Cy5-GlyGlyLeu conjugate as a dark blue solid. δ_H (CD3OD, 300MHz) 8.3(2H, 2t, vinylic), 7.8(2H, m, aromatic), 7.6 – 7.3(5H, m, aromatic), 6.6(1H, t, vinylic), 6.4(1H, d, vinylic), 6.2(1H, d, vinylic), 4.4(1H, m, leucine α -H), 4.1(2H, dd, -C4H8CH2CONH), 4.0(1H, d, Gly α -H), 3.8(2H, s, Gly α -H), 3.7(1H, d, Gly α -H), 3.6(3H, s, CH3N+), 2.4(2H, dd, CH2N), 2.0 – 1.5(9H, m, 4xCH2, Leu CH(CH3)2), 0.85(6H, s, Leu CH(CH3)2); δ_C (CD3OD, 75.45MHz) 177.5, 176.2, 174.3, 172.2, 156.6, 155.0, 145.9, 143.3, 143.1, 142.7, 142.1, 129.9, 128.0, 127.3, 126.9, 123.5, 121.2, 112.7, 110.8, 105.7, 104.1, 56.8, 55.8, 51.0, 45.2, 43.7, 42.9, 36.3, 31.4, 28.3, 27.8, 27.3, 26.1, 23.8, 22.2, 18.0, 13.1; λ_{max} 642nm.

b) Coupling of Dye-Linker Group to Nucleoside

Cy5-GlyGlyLeu-OH (3.1mg, 3.9 μ mol) and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (1.4mg, 4.7 μ mol) [Fluka] were weighed into a 1mL plastic tube. Anhydrous dimethylsulfoxide (50 μ L) [Aldrich] was then added, followed by diisopropylethylamine (0.74mg, 5.8 μ mol, 1 μ L) [Aldrich]. The reaction mixture was agitated and then allowed to stand at ambient temperature for 1 hour. A solution of 5-allylamino-2'-deoxyuridine-5'-triphosphate triethylammonium salt (3.8mg, 4.1 μ mol) in anhydrous dimethylsulfoxide (50 μ L) was added. The solution was agitated and then allowed to stand at ambient temperature. Monitoring of the reaction by reverse phase h.p.l.c showed the presence of a small amount of new material. The reaction mixture was diluted with 0.1M triethylammonium bicarbonate (TEAB) buffer and lyophilised to give a gummy residue. The residue was then re-dissolved in water (200 μ L) and then eluted through a preparative reverse phase h.p.l.c column (0.1M TEAB – Acetonitrile, 95:5 – 0:100 linear gradient over 30 minutes). Fractions containing the new material were collected and lyophilised. Further purification was performed by ion-exchange h.p.l.c to give the desired product as a blue foam after lyophilisation. δ_H (D2O, 300MHz) 7.9 – 7.1 (8H, m, aromatic), 6.4 – 5.9 (5H, m, vinylic), 6.1 (1H, obs, H-1'), 4.3 – 3.0(9H, m), 2.2(2H, m, H-2'), 2.0 – 1.5 (3H, m), 0.8 (6H, m); δ_P (D2O, 300MHz) -6.3, -11.1, -21.7; λ_{max} 644nm.

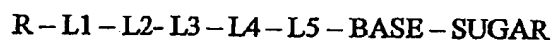
(vii) Cleavage of linker with Subtilisin

5 A solution of approximately 10µg of nucleoside in water (5µL) was mixed with 1 unit of subtilisin (2µl of 0.5U µl⁻¹ enzyme stock solution) in the appropriate buffer for the enzyme (193µl). Solutions were then held at enzyme optimum temperature for two hours and then centrifuged through 10,000D molecular weight size exclusion membranes [Amicon Microcon YM-10] to remove the enzyme. The appropriate subtilisin substrate reaction and standard control solutions were also prepared and
10 treated in the same manner. The filtrates were analysed using C18 reverse phase h.p.l.c (0.1%TFA/water : 0.042%TFA/Acetonitrile, 95:5 – 0:100 linear gradient). Successful cleavage of the linker was observed when new products at 4.2 and 25.2 minutes (starting material retention time = 24.2 minutes). The peak at 4.2 minutes contained only the nucleotide chromophore.

Claims

1. A nucleoside comprising a reporter moiety which also functions to limit
polymerase activity, characterised in that the reporter moiety is attached to the
5 nucleoside through a linkage group cleavable by a hydrolase enzyme, wherein said
hydrolase enzyme is selected from the group consisting of esterases, phosphatases,
peptidases, penicillin amidases, glycosidases and phosphorylases.

2. A compound of Formula I
10



(I)

15 wherein R is a reporter moiety

L1 and L5 are optional linkage groups each containing one or more
atoms comprising hydrocarbon chains which may also contain other
atoms such as N, O and S.

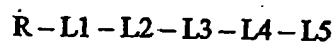
20

L2 and L4 are optional linkage groups comprising 1 or more amino
acid residues.

L3 is a linkage group that is susceptible to enzymic hydrolysis by a hydrolase enzyme,
25 wherein hydrolytic cleavage may be within the group or adjacent to the group and
characterised in that said hydrolase enzyme is selected from the group consisting of
esterases, phosphatases, peptidases, penicillin amidases, glycosidases and
phosphorylases.

30 3. A compound according to claim 2, wherein enzymic hydrolysis of the linkage
group L3 produces an unstable moiety which undergoes chemical hydrolysis.

4. A compound according to either claim 2 or 3, wherein the base comprises purines or pyrimidines and, in particular, any of the bases A, C, G, U and T or analogues thereof.
5. A compound according to any of claims 2 to 4, wherein the sugar comprises ribose or deoxy-ribose or analogues thereof.
6. A compound according to any of claims 2 to 5, wherein a mono-, di- or triphosphate group is attached to the sugar.
7. A compound according to claim 6, wherein a triphosphate group is attached to the sugar.
8. A compound according to any of claims 2 to 7, wherein the peptidase is selected from the group consisting of subtilisin, proteinase K, elastase, neprilysin, thermolysin, papain, plasmin, trypsin, enterokinase and urokinase.
9. A compound according to any of claims 2 to 8, wherein L3 is a peptide selected from the group consisting of alanine-alanine-alanine, alanine-alanine-leucine, glycine-leucine-serine, glycine-serine-alanine-alanine-leucine and glycine-alanine-glycine-leucine.
10. A compound as claimed in any of claims 2 to 9 wherein R is a fluorophore, selected from the group consisting of fluoresceins, rhodamines, coumarins, BODIPY® dyes, phenoxazine dyes, cyanine dyes, acridone dyes and squarate dyes.
11. A chemical intermediate of Formula II



(II)

wherein R is a reporter moiety

L1 and L5 are optional linkage groups each containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S.

5

L2 and L4 are optional linkage groups comprising 1 or more amino acid residues, and

L3 is a linkage group that is susceptible to enzymic hydrolysis by a peptidase enzyme.

10 12. A chemical intermediate according to claim 11, wherein L3 is selected from the group consisting of alanine-alanine-alanine, alanine-alanine-leucine, glycine-leucine-serine, glycine-serine-alanine-alanine-leucine and glycine-alanine-glycine-leucine.

15 13. A chemical intermediate according to either of claims 11 or 12, wherein R is selected from the group consisting of of fluoresceins, rhodamines, coumarins, BODIPY® dyes, phenoxazine dyes, cyanine dyes, acridone dyes and squarate dyes.

20 14. The compound 5-*N*-[*N*-(6-Fluorescein-5(and-6) carboxamidohexanoyl)-Gly-Gly-Leu- β -alanyl]-propargylamino-2'-deoxyuridine triphosphate.

15. A set of nucleotides characterised in that the set contains at least one compound according to claim 6.

25

16. A set of nucleotides as claimed in claim 15 comprising each of the four natural bases A, G, C and T and analogues thereof.

17. A set of nucleotides as claimed in either of claims 15 or 16 further comprising
30 at least two compounds according to claim 6 having different bases and characterised in that each compound has a different reporter moiety, R.

18. A set of nucleotides as claimed in any of claims 15 to 17 comprising four compounds according to claim 6 characterised in that each compound has a different base such that each of the bases A, G, C and T, or analogues thereof, are present and each of the four compounds has a reporter moiety which is distinguishable from the reporter moiety of each of the compounds having the other three bases.

19. A method for nucleic acid molecule sequencing comprising the steps of:

- a) immobilising a complex of a primer and a template to a solid phase
- b) incubating with a polymerase in the presence of a compound according to claim 6.

20. A method as claimed in claim 19 further comprising the steps of:

- c) detecting the incorporation of a compound according to claim 6
- d) incubating in the presence of hydrolase enzyme under suitable conditions for enzymatic cleavage of the enzyme-cleavable group L3.

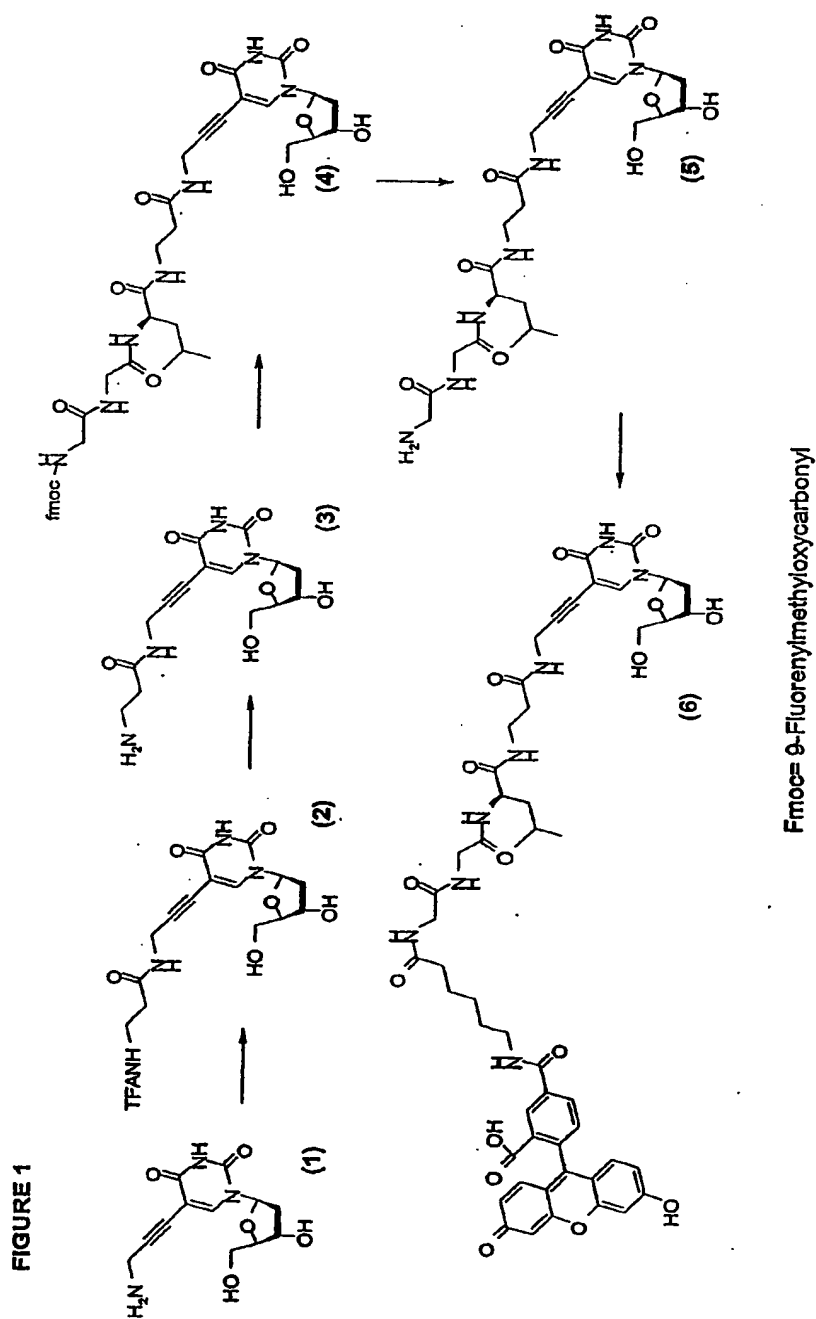
21. A method according to claim 20, wherein the hydrolase enzyme is selected from the group consisting of esterases, phosphatases, peptidases, penicillin amidases, glycosidases and phosphorylases.

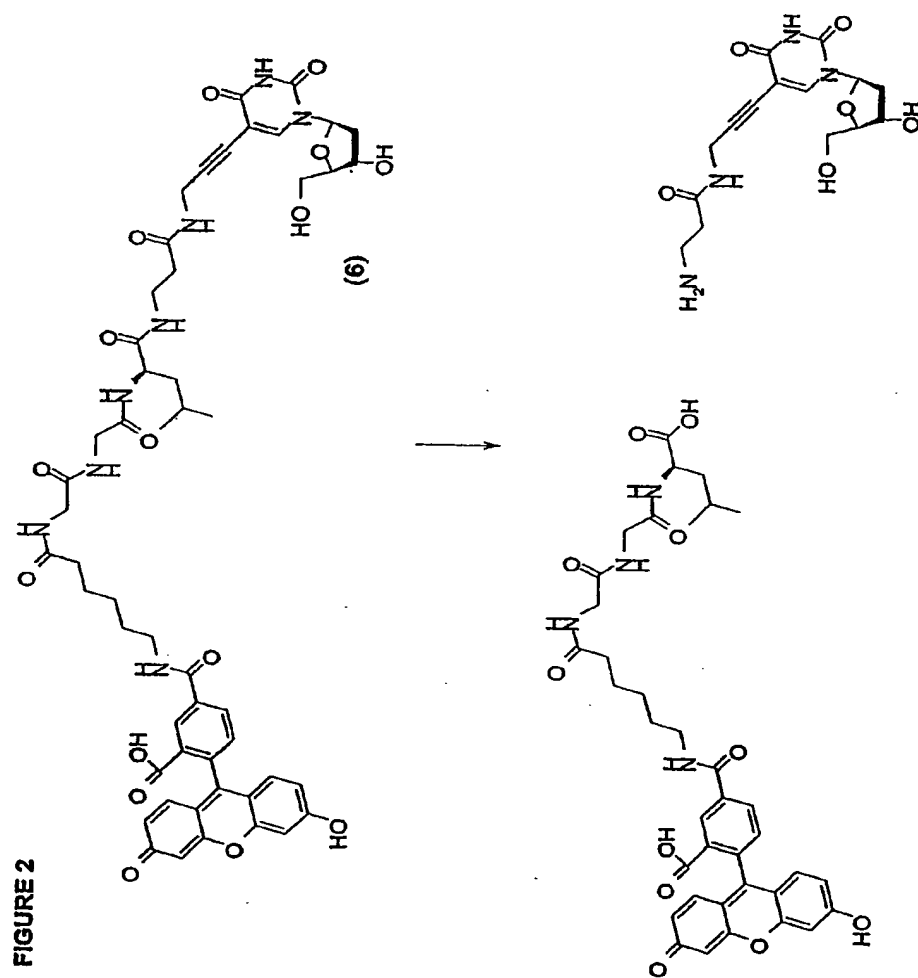
22. A method according to claim 21, wherein the peptidase is selected from the group consisting of subtilisin, proteinase K, elastase, neprilysin, thermolysin, papain, plasmin, trypsin, enterokinase and urokinase.

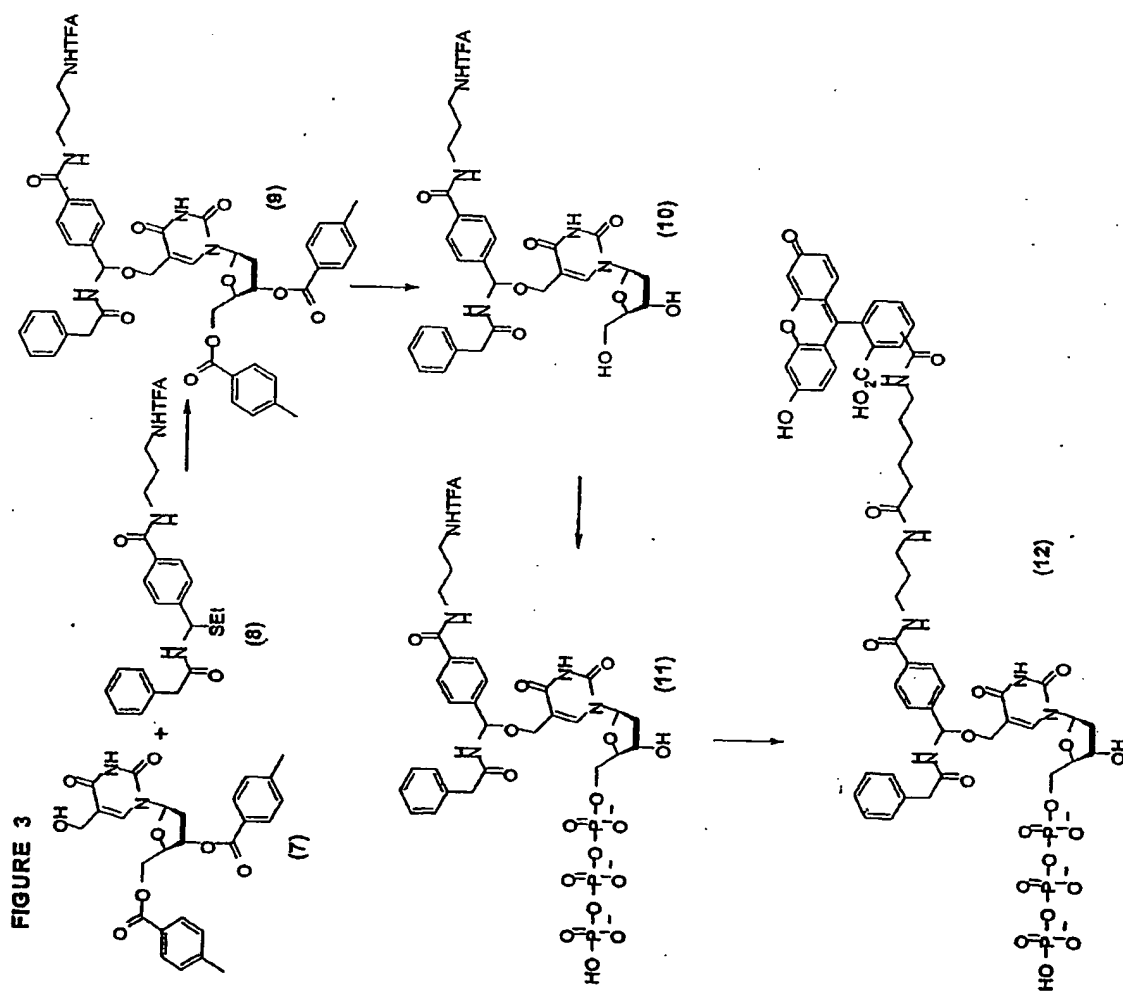
23. A method as claimed in any of claims 19 to 22 further comprising:

- e) repeating steps a)-d).

24. A method according to any of claims 19 to 23, wherein the incorporation of the compound is determined by the detection of a single reporter group attached to the compound.







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<170> PatentIn version 3.1

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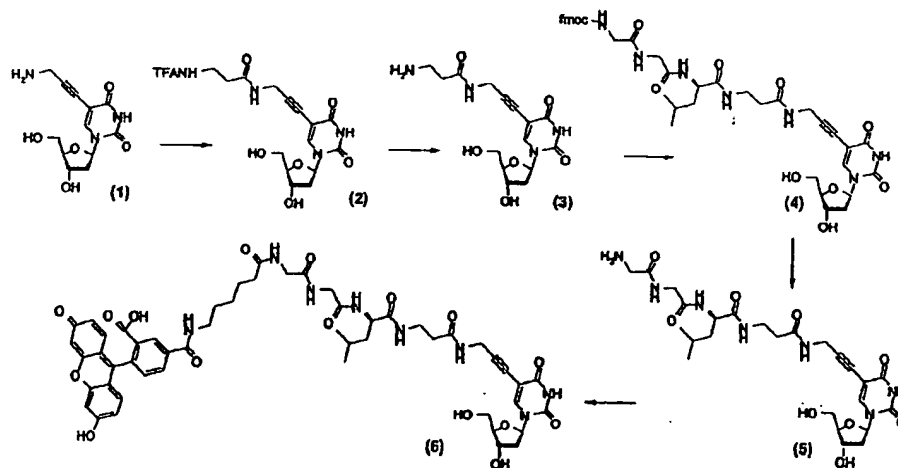
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LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
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[Continued on next page]

(54) Title: NUCLEOTIDE ANALOGUES AND THEIR USE FOR NUCLEIC ACID SEQUENCING



Fmoc= 9-Fluorenylmethoxycarbonyl

(57) Abstract: The invention relates to nucleosides comprising a reporter moiety which also functions to limit polymerase activity, characterised in that the reporter moiety is attached to the nucleoside through a linkage group cleavable by a hydrolase enzyme wherein the hydrolase enzyme is selected from the group consisting of esterases, phosphatases, peptidases, penicillin amidases, glycosidases and phosphorylases.

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BS, FI, FR, GB, GR, IH, IT, LU, MC, NL, PT, SE, SK,
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Internal application No
PCT/ep 02/05375

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C07H19/00 C12Q1/68 C07K1/107 C07K1/13 C09B1/36		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C07H C12Q C07K C09B		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, CHEM ABS Data, BEILSTEIN Data, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 850 949 A (PASTEUR INSTITUT) 1 July 1998 (1998-07-01) page 4, lines 27-29; page 11, line 13 page 14, line 11; compound 13, 14, 21-23; examples; claims 1-7	1
X	WO 88 10264 A (FLOREY HOWARD INST) 29 December 1988 (1988-12-29) claims 11, 13; figure 2A, compound B; page 17, lines 20-21	1-8, 10, 15, 16
X	WO 97 00967 A (BAYLOR COLLEGE MEDICINE) 9 January 1997 (1997-01-09) figures 17 and 18; page 12, line 23 - page 18, line 21	1-8, 10, 15-18
-/-		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 4 June 2003		Date of mailing of the international search report 21 Oct. 2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3018		Authorized officer Hennard, C.

INTERNATIONAL SEARCH REPORT

Internal application No

PCT/GB 02/05375

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 64437 A (ORCHID BIOCOMPUTER INC) 16 December 1999 (1999-12-16) figures	1
X	US 5 998 603 A (TENG KELLY ET AL) 7 December 1999 (1999-12-07) compound 16	1
P,X	WO 01 92284 A (AMERSHAM PHARM BIOTECH UK LTD ;PICKERING LEE (GB); ODEDRA RAJ (GB)) 6 December 2001 (2001-12-06) Figures 1, 2, 5-12; claims 1-11, 13-20; examples	1-8,10, 15-24
P,X	US 2001 049105 A1 (XIAO VIVIAN ET AL) 6 December 2001 (2001-12-06) figures 4, 5, 33-34	1-8,10, 15-18
P,X	WO 01 94609 A (WILLIAMS JOHN G K ;SHEAFF PAMELA (US); CHEN JIYAN (US); DRANEY DAN) 13 December 2001 (2001-12-13) page 3, line 9 - page 6, line 10; figure 12, compound 56; figures 4-6	1
X	CONFALONE P N: "The use of heterocyclic chemistry in the synthesis of natural and unnatural products" JOURNAL OF HETEROCYCLIC CHEMISTRY, HETEROCORPORATION. PROVO, US, vol. 27, 1990, pages 31-46, XP002159718 ISSN: 0022-152X page 40-43	1-8,10, 15-24
X	SAUER M ET AL: "DETECTION AND IDENTIFICATION OF SINGLE DYE LABELED MONONUCLEOTIDE MOLECULES RELEASED FROM AN OPTICAL FIBER IN A MICROCAPILLARY: FIRSTSTEPS TOWARDS A NEW SINGLE MOLECULE DNA SEQUENCING TECHNIQUE" PHYSICAL CHEMISTRY CHEMICAL PHYSICS, ROYAL SOCIETY OF CHEMISTRY, CAMBRIDGE, GB, vol. 1, no. 10, 15 May 1999 (1999-05-15), pages 2471-2477, XP000854111 ISSN: 1463-9076 page 2474, figure2;	1-8,10, 15-24

-/-

INTERNATIONAL SEARCH REPORT

Internat. application No

PCT/GB 02/05375

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PROBER J M ET AL: "A SYSTEM FOR RAPID DNA SEQUENCING WITH FLUORESCENT CHAIN-TERMINATING DIDEOXYNUCLEOTIDES" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 238, no. 4825, 16 October 1987 (1987-10-16), pages 336-341, XP000604017 ISSN: 0036-8075 page 338, figure 2A</p>	1-8, 10, 15-17
X	<p>DVORSKY DRAHOMIR ET AL: "CATIONIZATION AGENT FOR REACTIVE DYEING, SOFTENING, FIXATION AND ANTISTATIC FINISHING OF TEXTILES" CHEMICAL ABSTRACTS + INDEXES, AMERICAN CHEMICAL SOCIETY, COLUMBUS, US, vol. 110, no. 12, 12 June 1989 (1989-06-12), page 100, XP000056240 ISSN: 0009-2258 page 2224, compound 4</p>	1-8, 10
P, X	<p>FINN, PATRICK J. ET AL: "Synthesis and application of charge-modified dye-labeled dideoxynucleoside-5'-triphosphates to "direct-load" DNA sequencing--" NUCLEIC ACIDS RESEARCH (2002), 30(13), 2877-2885, 2002, XP002243107 the whole document</p>	1-8, 10, 15-24
X	<p>WO 95 03429 A (UNIV UTAH RES FOUND) 2 February 1995 (1995-02-02) the whole document</p>	11
X	<p>EP 1 096 257 A (SAUER MARKUS ;WOLFRUM JUERGEN (DE)) 2 May 2001 (2001-05-02) the whole document</p>	11

INTERNATIONAL SEARCH REPORT

Inte al application No.
PCT/GB 02/05375

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-10, 14-24

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-10, 14-24

The first invention concerns a nucleoside or nucleotide bearing a reporter moiety cleavable by a hydrolase (peptidase).

2. claims: 10-13

The second invention concerns a peptide bearing a reporter moiety.

INTERNATIONAL SEARCH REPORT

 Internat
 application No
 PCT/GB 02/05375

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0850949	A	01-07-1998	FR 2703052 A1 EP 0850949 A2 CA 2158975 A1 EP 0690928 A1 WO 9423064 A1 JP 8508473 T US 5798210 A	30-09-1994 01-07-1998 13-10-1994 10-01-1996 13-10-1994 10-09-1996 25-08-1998
WO 8810264	A	29-12-1988	AT 113059 T AU 1990988 A WO 8810264 A1 CA 1340032 C DE 3851889 D1 DE 3851889 T2 EP 0366685 A1 JP 2504144 T JP 2828642 B2 NZ 225176 A US 5552540 A	15-11-1994 19-01-1989 29-12-1988 08-09-1998 24-11-1994 13-04-1995 09-05-1990 29-11-1990 25-11-1998 26-09-1990 03-09-1996
WO 9700967	A	09-01-1997	US 5614386 A US 5861287 A US 5728529 A US 5994063 A AU 699939 B2 AU 6288696 A CA 2225531 A1 EP 0833936 A1 WO 9700967 A1	25-03-1997 19-01-1999 17-03-1998 30-11-1999 17-12-1998 22-01-1997 09-01-1997 08-04-1998 09-01-1997
WO 9964437	A	16-12-1999	US 6287821 B1 AU 4438099 A WO 9964437 A1	11-09-2001 30-12-1999 16-12-1999
US 5998603	A	07-12-1999	US 5608046 A US 5965721 A US 6320040 B1 AU 3892395 A WO 9610030 A1 US 2003045705 A1 US 2003139586 A1	04-03-1997 12-10-1999 20-11-2001 19-04-1996 04-04-1996 06-03-2003 24-07-2003
WO 0192284	A	06-12-2001	AU 6249501 A CA 2408143 A1 EP 1287012 A1 WO 0192284 A1	11-12-2001 06-12-2001 05-03-2003 06-12-2001
US 2001049105	A1	06-12-2001	US 6322980 B1 US 6514700 B1 AU 4497200 A CA 2368581 A1 EP 1180112 A1 JP 2002542811 T US 2002146726 A1 US 2002142329 A1 US 2002150927 A1 WO 0066607 A1 US 2001051340 A1	27-11-2001 04-02-2003 17-11-2000 09-11-2000 20-02-2002 17-12-2002 10-10-2002 03-10-2002 17-10-2002 09-11-2000 13-12-2001